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Induction of MIF synthesis and secretion by tubular epithelial cells: A novel action of angiotensin II

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Induction of MIF synthesis and secretion by tubular epithelial cells: A novel action of angiotensin II.

Background. Angiotensin II (Ang II) plays an important role in the development of renal injury through its vasoactive and proinflammatory activities. We investigated whether some of the effects of Ang II could be mediated through the production of macrophage migration inhibitory factor (MIF).

Methods. Groups of rats underwent sham surgery (sham), subtotal nephrectomy (STNx), or STNx plus treatment with irbesartan. Renal tissue was examined 12 weeks postsurgery for MIF mRNA expression and leukocyte accumulation. To determine whether Ang II had a direct effect on MIF production, mRNA synthesis and protein secretion were examined in proximal tubular epithelial (NRK52E and MCT) cell lines.

Results. MIF mRNA was strongly expressed in 5.4% ± 1.1% (mean ± SD) of cortical tubules of sham-operated rats. This was significantly up-regulated in STNx rats (44.9% ± 22.6%) and was abrogated by administration of irbesartan (2.8% ± 2.4%). STNx resulted in significant glomerular and interstitial accumulation of macrophages and T cells, which correlated with glomerular and tubular MIF mRNA expression, respectively. In vitro studies of tubular epithelial cells revealed that Ang II caused a twofold increase in MIF mRNA expression in NRK52E and MCT cells, which was abrogated by irbesartan. In addition, Ang II induced a rapid release of 50% of MIF protein from NRK52E cells within 20 minutes.

Conclusion. This study has demonstrated that Ang II up-regulates MIF mRNA production and MIF protein secretion by tubular epithelial cells. Ang II may promote accumulation and activation of interstitial leukocytes via induction of MIF synthesis and secretion in renal tubular epithelial cells. This may be an important mechanism by which Ang II mediates renal injury.

Key words: MIF, angiotensin, subtotal nephrectomy.

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The renin-angiotensin system (RAS) has been implicated in the pathogenesis of renal damage. Inhibition of the RAS by angiotensin-converting enzyme inhibitors (ACEI) or angiotensin subtype 1 receptor antagonists (AT_1RA) significantly attenuates the decline in renal function associated with diabetic [1, 2] and nondiabetic [3, 4] renal diseases. As well as regulating glomerular hypertension, angiotensin II (Ang II) has a range of nonhemodynamic effects that are important in mediating renal damage.

The proinflammatory actions of Ang II, including the induction of growth factors [5], cytokines [6], chemokines [7–10], and adhesion molecules [11–15] have been revealed. Ang II also promotes mononuclear cell recruitment and proliferation, as well as increased extracellular matrix (ECM) accumulation and subsequent renal injury. A pathogenic role of Ang II in immune mediated renal disease has been demonstrated in two rodent models: immune complex glomerulonephritis and antiglomerular basement membrane nephritis [8, 16–18].

Glomerular and interstitial macrophage and T-cell accumulation is a feature of most types of renal disease [19] and precedes glomerulosclerosis and renal fibrosis. The accumulation of macrophages occurs through a process of recruitment and proliferation of circulating monocytes. Macrophage migration inhibitory factor (MIF), a proinflammatory cytokine, is an important regulator of macrophage accumulation at sites of tissue injury [20].

MIF, a 12.5 kD protein, was originally described as a lymphocyte product able to inhibit macrophage migration and regulate the delayed-type hypersensitivity response [21, 22]. Since this original description, it is known that MIF is a unique molecule that regulates inflammation [23], cell proliferation [24], differentiation [25], and angiogenesis [26]. MIF is up-regulated in glomerular and tubular epithelial cells in human proliferative glomerulonephritis and renal transplant rejection, where the de-

gree of up-regulation corresponds to histologic damage and loss of renal function [20, 27]. In the diseased kidney, infiltrating macrophages and T cells express MIF, but the renal parenchymal cells are the major site of MIF expression [20, 28]. The pathogenic role of MIF has been illustrated in a rat model of crescentic antiglomerular basement membrane nephritis, where neutralizing antibodies, administered prophylactically or therapeutically, reduce renal damage [29, 30].

Because Ang II has been implicated in promoting immune mediated renal injury and MIF mediates leukocyte accumulation and activation, we hypothesized that Ang II may cause renal inflammation via the production of MIF by tubular epithelial cells. To evaluate this, we examined the effect of Ang II blockade on MIF expression and leukocytic infiltration in rats after subtotal nephrectomy (STNx). The direct effects of Ang II on MIF production and release were determined using cultured rodent tubular epithelial cells.

METHODS

Experimental protocol for animal study

Renal tissues from a previously published study were examined [31]. Eight-week-old Sprague-Dawley rats (220 to 280 g) underwent STNx (right nephrectomy followed by infarction of approximately two thirds of the left kidney) or sham surgery (laparotomy and manipulation of both kidneys, $N = 8$). Rats undergoing STNx were randomly allocated to receive no drug therapy ($N = 8$) or irbesartan, an AT_1RA, for 12 weeks at a dose of 15 mg/kg per day by gavage ($N = 8$). Animals were sacrificed after 12 weeks following measurement of systolic blood pressure, plasma creatinine, and 24-hour urinary protein and creatinine. Remnant kidneys were removed and fixed in 10% formalin and renal tissue examined.

Reagents

Antibodies used for immunohistochemistry were ED-1, mouse antirat CD68 monoclonal antibody (mAb), which labels most monocytes and macrophages [32] (Serotec, Kidlington, Oxford, UK); W3/13, mouse antirat CD43 (leucosialin) mAb, which labels T lymphocytes and some granulocytes [33]; polyclonal rabbit anti-MIF serum [23]; horseradish peroxidase (HRP)-and alkaline phosphatase (AP)-conjugated goat antimouse IgG (Dako, Carpinteria, CA, USA); mouse or rabbit peroxidase antiperoxidase complexes (PAP, Dako); and mouse alkaline phosphatase antialkaline phosphatase complexes (APAAP, Dako). Mouse anti-MIF mAb (III.D.9) [28] and mouse antirat CD8 mAb (OX-8) [34] were used for flow cytometry analysis using fluorescence-activated cell sorter (FACS) after labeling with Alexa 488 fluorescence dye (Alexa 488 Protein Labeling Kit A-10235, Molecular Probes, Eugene, Oregon, USA). A cloned 325 base pair (bp) fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a cloned 441 bp of rat MIF were linearized and digoxigenin (DIG)-labeled using T7-RNA polymerase to produce cRNA sense or antisense probes for Northern Blotting and in situ hybridization according to the manufacturer's protocol (Roche, Mannheim, Germany). Ang II was purchased from Calbiochem (Melbourne, Victoria, Australia). Irbesartan was a gift from Bristol-Myers Squibb Pharmaceuticals (Princeton, NJ, USA).

In situ hybridization

In situ hybridization was performed on 4 μm paraffin sections of formalin-fixed tissue using a modified version of a standard protocol [35]. Briefly, sections were incubated with 0.2 mol/L HCl for 20 minutes, followed by 0.3% Triton X-100 for 15 minutes, and then digested for 20 minutes with 10 μg/mL proteinase K (Roche) at 37°C. After fixation in 4% paraformaldehyde for 10 minutes, sections were incubated for 1 hour in hybridization buffer, then hybridized with 0.1 ng/μL DIG-labeled antisense or sense MIF cRNA probe overnight at 37°C. Hybridization buffer contained 50% deionized formamide, 4 × standard saline citrate (SSC) solution, 1 mg/mL salmon sperm DNA (Sigma, Castle Hill, NSW, Australia), and 1 mg/mL yeast tRNA. The next day, sections were washed in 2 × SSC then 0.1 × SSC at 42°C for 20 minutes, and the bound DIG-labeled probe detected using AP-conjugated sheep anti-DIG F(ab) fragment, and developed with the chromogenic agents, nitro blue tetrazolium (NBT) and X phosphate (Roche). Cortical tubules showing a strong signal for MIF mRNA were scored by evaluating 20 high power fields (×400) randomly selected in the renal cortex in the animal tissues and counting the percentage of MIF-positive tubules. Approximately 1000 tubules were scored for each animal. Fifty glomeruli per animal were assessed and the number of MIF mRNA+ cells per glomerular cross-section (gcs) counted.

Immunohistochemistry

Immunostaining for MIF and leukocytes was performed on 4 μm formalin-fixed sections was performed as previously described [28, 36]. Briefly, sections for ED-1 staining were microwaved in citrate buffer pH 6.0 for 12 minutes and then cooled on ice. Sections for MIF or W3/13 staining were not microwaved. Sections were blocked with 10% normal sheep serum and then incubated with ED-1, W3/13, or rabbit anti-MIF antibodies in 1% bovine serum albumin (BSA) overnight at 4°C. The following day, endogenous peroxidase was inactivated with 0.3% H_2O_2 in methanol, and then sections were incubated with HRP-conjugated goat antimouse or antirabbit immunoglobulin G (IgG), followed by mouse or rabbit PAP and developed with diaminobenzamide (Sigma) to produce a brown color. For two-color immunostaining, sections were first stained for MIF as de-

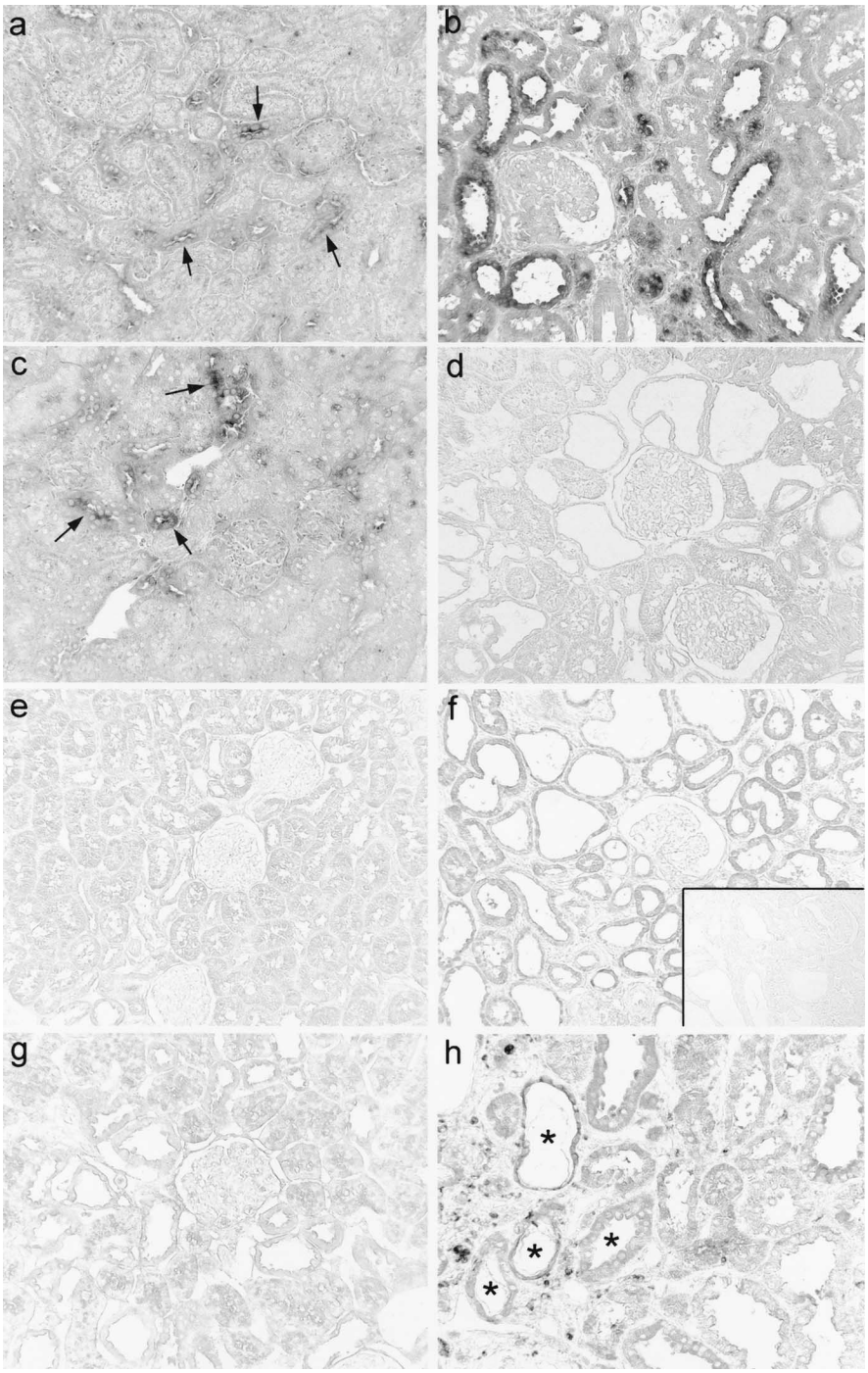

Fig. 1. Macrophage migration inhibitory factor (MIF) mRNA and protein expression following subtotal nephrectomy (STNx). MIF expression was detected by in situ hybridization and MIF protein by immunohistochemistry staining. (*a*) Kidney from a sham-operated rat showing very weak expression of MIF mRNA in most tubules, with strong MIF mRNA expression in a small number of tubules (arrows). (*b*) Kidney from a rat 12 weeks after undergoing STNx showing strong MIF mRNA expression in many tubules, particularly dilated and damaged tubules. (*c*) Kidney from a rat 12 weeks after STNx and irbesartan treatment showing only a small number tubules with strong MIF mRNA expression (arrows). (*d*) No signal was seen in an area of tissue damage in STNx using a MIF sense probe. (*e*) Very weak immunostaining for MIF protein is seen in most tubules in a sham-operated rat. (*f*) Strong MIF immunostaining is seen in many dilated and damaged tubules at 12 weeks after STNx. Inset shows a lack of staining in a damaged STNx kidney using an isotype-matched control antibody. (*g*) Kidney from a rat 12 weeks after STNx and irbesartan treatment showing only weak tubular MIF immunostaining, similar to sham-operated rat. (*h*) Two-color immunostaining showing focal interstitial accumulation of ED-1+ macrophages (blue) in an area with damaged tubules showing strong MIF protein staining (*), whereas the area to the right shows only weak tubular MIF immunostaining and no macrophage accumulation. Magnification (a) to (g), ×160; (h), ×250.

scribed above, microwaved to prevent antibody cross-reactivity [36], and then stained for macrophages using the ED-1 antibody followed by AP-conjugated goat anti-mouse IgG and mouse APAAP and developed with Fast Blue BB salt (Ajax Chemicals, Melbourne, Australia) to produce a blue color.

The number of ED-1 and W3/13 labeled cells was counted under high power (×400) in 25 glomeruli for each animal and was expressed as the number of labeled cells/gcs. To assess tubulointerstitial staining, the number of labeled interstitial cells was counted on 50 high power fields (×400) for each animal by means of a 0.02 mm^2 graticule fitted in the eyepiece of the microscope and expressed as cells per mm^2.

Cell culture

A rat tubular epithelial cell line (NRK52E) was obtained from the American Type Culture Collection

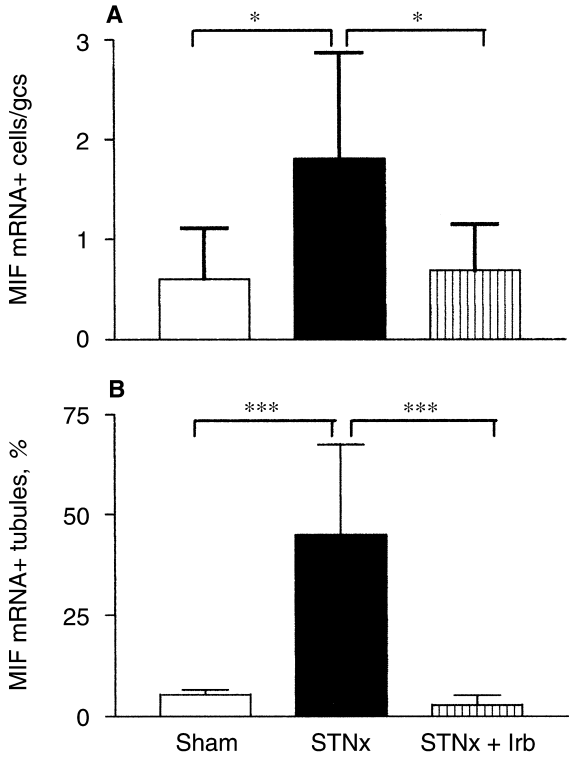

Fig. 2. Quantification of macrophage migration inhibitory factor (MIF) mRNA expression following subtotal nephrectomy (STNx). (*A*) The number of MIF mRNA+ cells per glomerular cross-section (gcs). (*B*) The percentage of cortical tubules with strong MIF mRNA expression. Sham surgery (sham), STNx, STNx + irbesartan treatment (STNx + Irb). Data are mean ± SD. $*P < 0.05$; $***P < 0.001$ by ANOVA with Bonferroni's post-test comparison.

(ATCC, Manassas, VA, USA) and a mouse proximal tubular epithelial cell line (MCT) was a gift from Professor Eric Neilson [37]. Cells were maintained in low glucose (1 g/L, NRK52E) or high glucose (4.5 g/L, MCT) Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 100 IU/mL penicillin-streptomycin, and 2 mmol/L glutamine in a humidified incubator at 37°C.

Northern blotting

Semiconfluent flasks of NRK52E cells were starved in 0.5% FCS/DMEM for 24 hours. Flasks of cells were then left unstimulated or stimulated for 6 hours with 10^{-5} to 10^{-7} mol/L Ang II with or without preincubation for 1 hour with 10^{-4} mol/L irbesartan. Semiconfluent flasks of MCT cells were starved in serum-free DMEM for 24 hours, then left unstimulated or stimulated for 6 hours with 10^{-7} mol/L Ang II with or without preincubation for 1 hour with 10^{-4}mol/L irbesartan.

For all experiments total cellular RNA was extracted from cell pellets using TRIzol Reagent (GIBCO BRL, Gaithersburg, MD, USA). Denatured RNA (15 ug) was size fractionated by electrophoresis on a 1.2% agarose gel, then capillary blotted onto a nylon membrane (Magna Nylon Transfer Membrane; Micron Separation, Inc., Westborough, MA, USA). The membrane was hybridized overnight with a DIG-labeled MIF or GAPDH cRNA probe. Bound probes were detected with AP-conjugated sheep anti-DIG Fab fragments (Roche) and developed with CPD star (Roche)–enhanced chemiluminescence, which was captured on Kodak BMR film. Densitometric analysis used the Gel Pro Analyzer program (Media Cybernetics, Silver Spring, MD, USA). Each experiment contained three to five replicates of flasks. All experiments were performed at least twice and the combined results analyzed.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from NRK52E and MCT cells and from normal rat and mouse kidney using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA (5 μg/sample) was reverse transcribed using oligo-dT(15) and Superscript II Reverse Transcriptase (Invitrogen) for 50 minutes at 42°C. The cDNA was amplified in a 50 μL PCR reaction containing 2.5 U Platinum Taq Polymerase (Invitrogen), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L deoxynucleotide triphosphate (dNTP) mix, 25 pmol oligonucleotide primers, 20 mmol/L Tris-HCl, pH 8.4, for 35 cycles on a GeneAmp PCR System 2400 (Perkin-Elmer, Inc., Norwalk, CT, USA). Samples were initially heated to 94°C for 2 minutes and then went through 35 cycles of 94°C for 30 seconds, 57°C for 60 seconds, and 72°C for 120 seconds, with a final extension step at 72°C for 7 minutes. The primers for mouse and rat AT1a were 5′-agcatcatctttgtggtggg-3′ and 5′-ttcgtagacaggcttgagtg-3′ (accession S37484) and primers for rat and mouse AT1b were 5′-tccaagatgactgccccaag-3′ and 5′-gttatctgaagggcggtagg-3′ (accession S37491). The PCR products were visualized by agarose gel electrophoresis and viewed under ultraviolet illumination.

Flow cytometry

To examine intracellular MIF protein expression, NRK52E cells were starved in 0.5% FCS/DMEM for 24 hours in 6-well plates. Cells were then stimulated with Ang II 10^{-5} mol/L for 0 minutes, 10 minutes, 20 minutes, 1 hour, or 2 hours (some cells were treated with 10^{-4} mol/L irbesartan for 1 hour before Ang II addition). After incubation, cells were removed by brief trypsinization, fixed in 1% paraformaldehyde for 30 minutes, and permeabilized with 0.1% saponin in 1% FCS/phosphate-buffered saline (PBS). Permeabilized cells were incubated with Alexa 488–labeled III.D.9 mouse anti-MIF mAb or Alexa 488–labeled OX-8 mAb (control) diluted in 0.1% saponin in 1% FCS/PBS for 30 minutes in the dark at 4°C. After incubation, cells were washed and analyzed on a flow cytometer connected to a data acquisi-

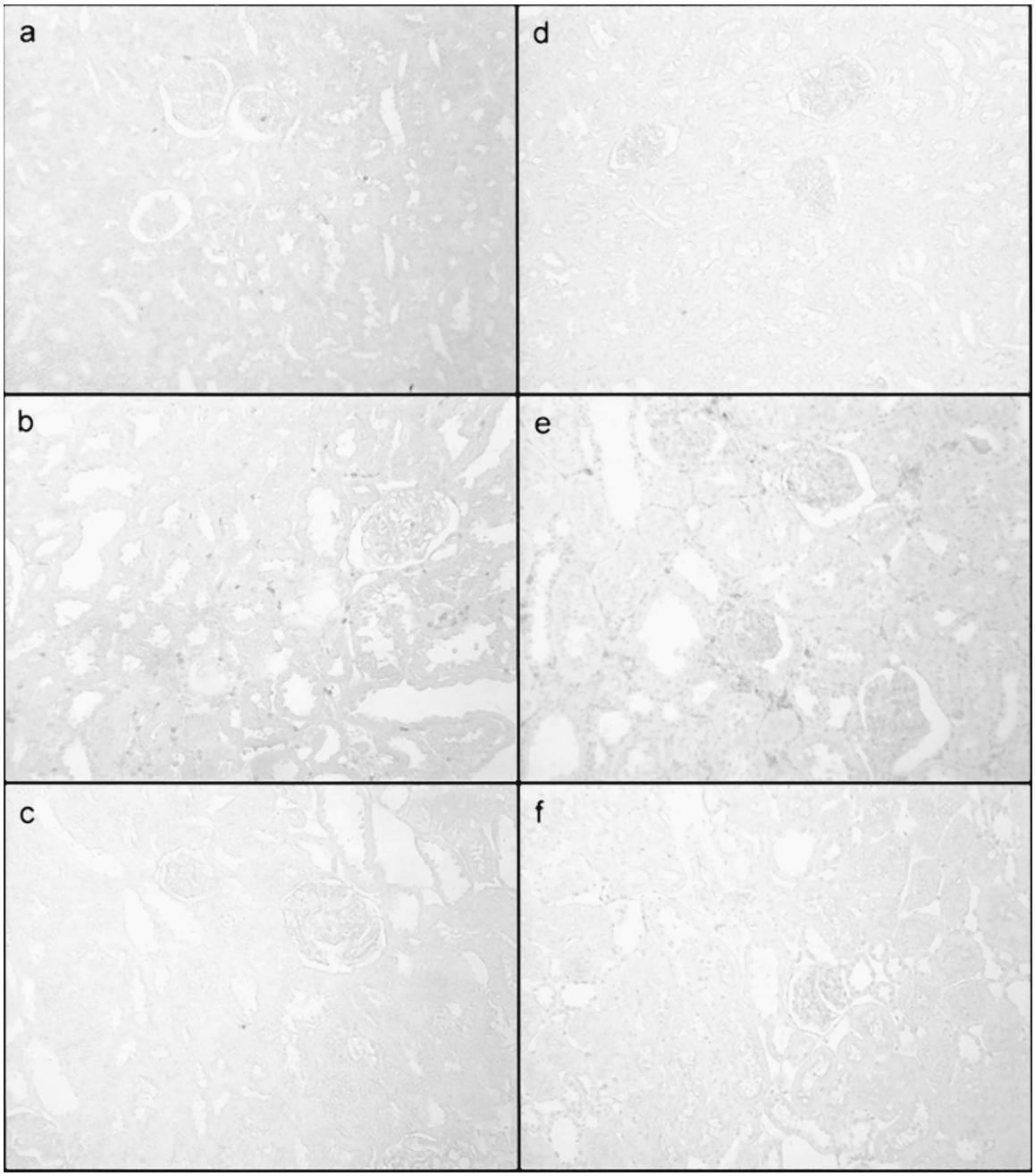

Fig. 3. Immunostaining of leukocytes in subtotal nephrectomy (STNx). ED-1+ macrophages (gray) in the kidney of (*a*) sham-operated rat, (*b*) week 12 following STNx in untreated rat, and (*c*) week 12 following STNx in rat treated with irbesartan. W3/13 + T cells (gray) in the kidney of (*d*) sham-operated rat, (*e*) week 12 following STNx in untreated rat, and (*f*) week 12 following STNx in rat treated with irbesartan. Magnification, ×160.

tion system. The cells were illuminated by an argon ion laser and fluorescence was measured. The viable cell population was gated from debris on the basis of forward angle and 90° angle light scatter. A fluorescence histogram of approximately 10,000 cells was obtained for each cell sample and the mean channel fluorescence (MCF) measured. For each experiment, the average MCF was calculated for unstimulated cells labeled with III.D.9 and assigned a value of 100%. The average MCF at each time point was expressed as a percentage of the unstimulated value. Each experiment contained two to three replicates at each time point. Three separate experiments were performed and the combined results were analyzed.

Western blotting

To examine intracellular MIF protein expression, semiconfluent flasks of NRK52E and MCT cells were lysed in 50 mmol/LTris-HCl, pH 7.4, 150 mmol/L NaCl,

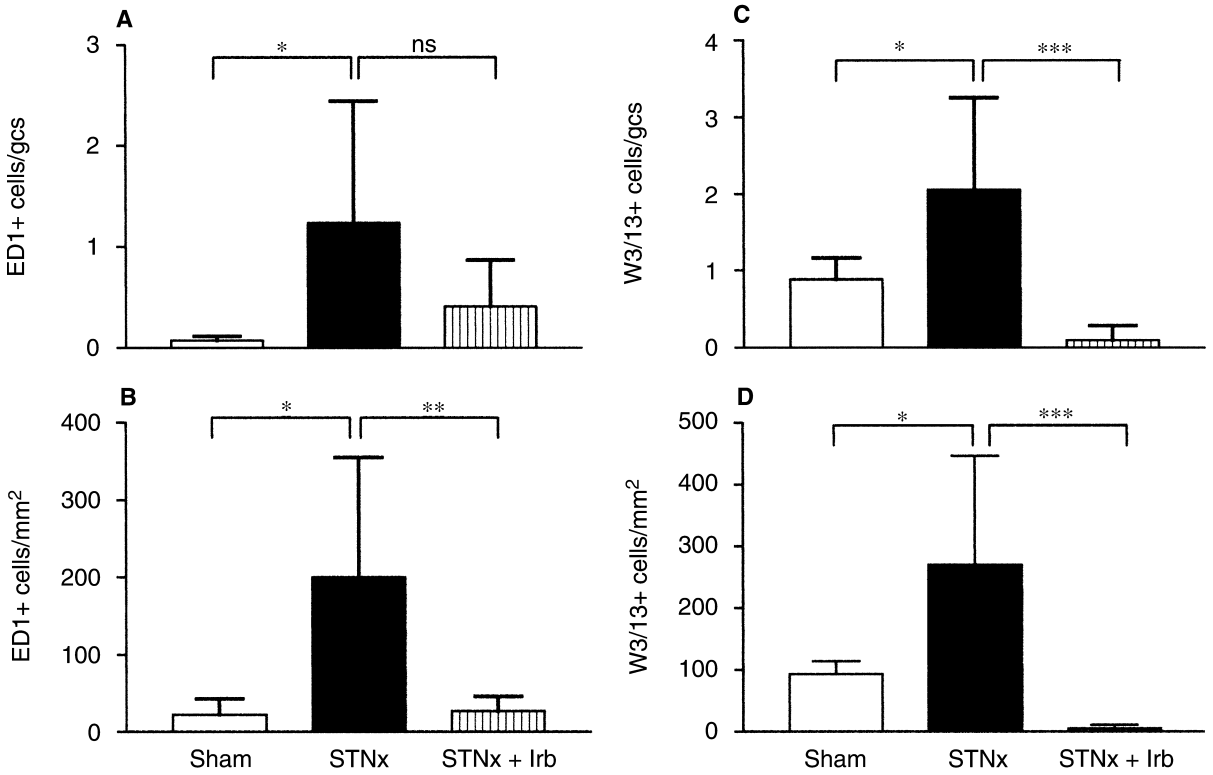

Fig. 4. Quantification of leukocytic infiltrate following subtotal nephrectomy (STNx). The number of (*A*) glomerular ED-1+ macrophages, (*B*) tubulointerstitial ED-1+macrophages, (*C*) glomerular W3/13 + T cells, and (*D*) tubulointerstitial W3/13 + T cells. Sham surgery (sham), STNx, STNX + irbesartan treatment (STNx + Irb). Data are mean ± SD. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by ANOVA with Bonferroni's post-test comparison.

1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS) containing a protease inhibitor cocktail (Sigma). Cell lysates were centrifuged and the supernatants collected and loaded onto 15% polyacrylamide gels containing SDS and run under reducing conditions. Lysate samples (approximately 50 μg) were separated by electrophoresis and then transferred to Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Castle Hill, NSW, Australia). MIF protein was detected as previously described [38]. Briefly, after blocking in 5% skim milk powder, membranes were incubated for 1 hour with rabbit anti-MIF serum (1:1000). After washing, membranes were incubated for 1 hour in HRP-conjugated antirabbit IgG (Silenus, Melbourne, Victoria, Australia), and MIF protein detected using an enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL, USA). Chemiluminescent emissions were captured on Kodak BMR film.

To examine MIF protein secretion, semiconfluent NRK52E cells were starved in 0.5% FCS/DMEM for 24 hours in 6-well plates. Cells were then stimulated with 10^{-5} mol/L Ang II for 20 minutes or left unstimulated. Cell culture supernatant (1 mL) was collected and concentrated by immunoprecipitation using anti-MIF antibodies. Briefly, 50 μL rabbit anti-MIF serum was incubated with 500 μL protein A sepharose gel (Amersham) for 1 hour at 4°C, then the gel was washed by centrifugation three times in PBS. Cell culture supernatants were then incubated with the gel for 2 hours at 4°C. After washing three times, the gel was re-suspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and boiled (5 minutes) to solubilize the bound MIF in the sample buffer. The immunoprecipitated MIF was run on a 15% polyacrylamide gels containing SDS and detected by Western blotting as described above.

Statistics

Comparisons were performed by Student *t* test or by analysis of variance (ANOVA) with Bonferroni's post-test comparison. Correlations were performed using Pearson's correlation coefficient. All analyses were performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Irbesartan suppresses renal injury in STNx

STNx rats developed elevated systolic blood pressure, proteinuria, and a reduced glomerular filtration rate (GFR)—all of which were normalized by irbesartan

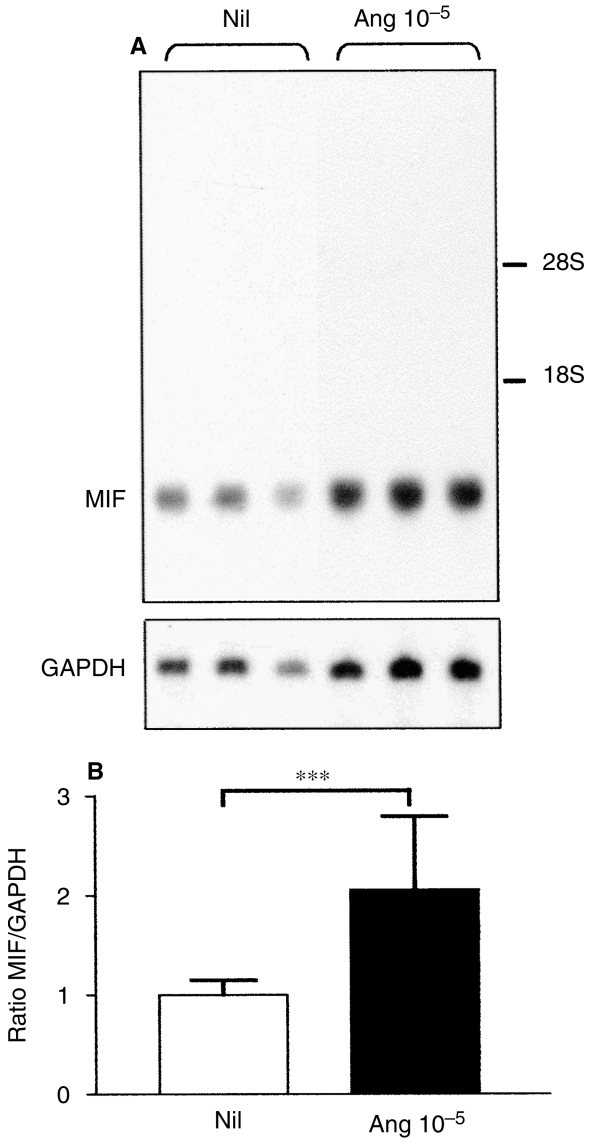

Fig. 5. Angiotensin II (Ang II) stimulates macrophage migration inhibitory factor (MIF) mRNA expression in NRK52E cells. (*A*) Northern blot in which NRK52E cells were either left unstimulated (Nil) or stimulated with 10^{-5} mol/L Ang II for 6 hours. Blots were probed for MIF and then reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (*B*) Results of densitometric analysis of the combined results of three experiments are shown. Data are mean ± SD. ***$P <$ 0.001 by Student *t* test.

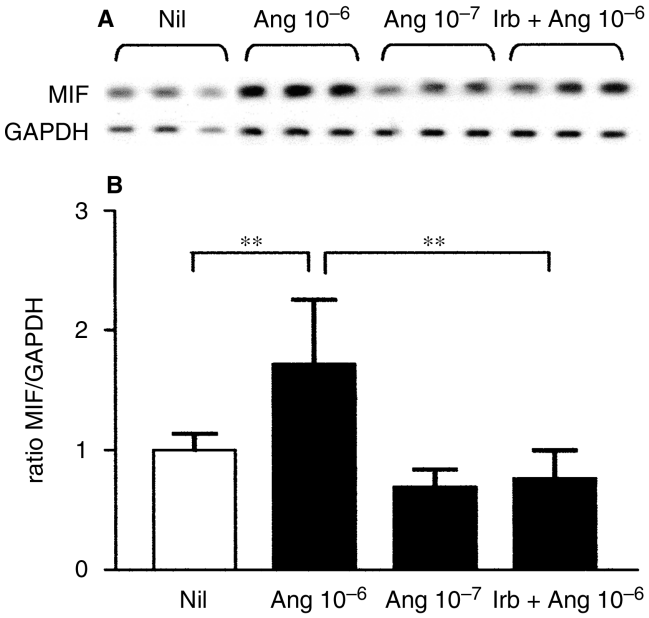

Fig. 6. Irbesartan (Irb) blocks angiotensin II (Ang II) stimulation of macrophage migration inhibitory factor (MIF) mRNA expression in NRK52E cells. (*A*) Northern blot in which NRK52E cells were either left unstimulated (Nil), stimulated with 10^{-6} or 10^{-7} mol/L Ang II for 6 hours or incubated with 10^{-4} mol/L Irb for 1 hour prior to stimulation with Ang II 10^{-6} mol/L (Irb + Ang 10^{-6}) for 6 hours. Blots were probed for MIF and then reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (*B*) Results of densitometric analysis of the combined results of three experiments are shown. Data are mean ± SD. **$P < 0.01$ by ANOVA with Bonferroni's post-test comparison.

treatment [31]. STNx rats developed glomerular changes, consisting of hyalinosis or sclerosis, and tubulointerstitial damage, consisting of inflammation, fibrosis, tubular atrophy, and dilatation, as well as cast formation. Both glomerulosclerosis and tubulointerstitial damage were significantly reduced by irbesartan treatment [31]. Sham-operated animals were not different to normal controls.

MIF mRNA expression

In situ hybridization revealed very weak MIF mRNA expression in most tubular epithelial cells in sham-operated rats, with a small number of tubules showing a strong signal for MIF mRNA. The percentage of tubules with strong MIF mRNA expression increased from 5.4% ± 1.1% (mean ± SD) in sham-operated rats to 44.9% ± 22.6% in STNx rats but was prevented by daily administration of irbesartan (Figs. 1 and 2). Only a few glomerular cells expressed MIF mRNA, which increased nearly twofold in STNx rats. Irbesartan prevented the increase in MIF mRNA expression in the glomerulus (Figs. 1 and 2). Interstitial cells also expressed MIF mRNA following STNx.

Macrophage and T-cell infiltrate

Significant infiltration of ED-1+ macrophages and W3/13+ T cells into the glomerulus and tubulointerstitium was evident in STNx rats (Figs. 3 and 4). Two-color immunostaining showed that interstitial macrophage accumulation was mainly localized to areas with strong tubular MIF expression (Fig. 1h). Treatment with irbesartan completely ameliorated the leukocytic infiltrate (Figs. 3 and 4). The number of glomerular MIF mRNA+ cells correlated with glomerular macrophage ($r^2 = 0.80$, $P < 0.0001$) and T-cell infiltration ($r^2 = 0.71$, $P < 0.0001$). In addition, the percentage of tubules with strong MIF mRNA expression correlated with interstitial macro-

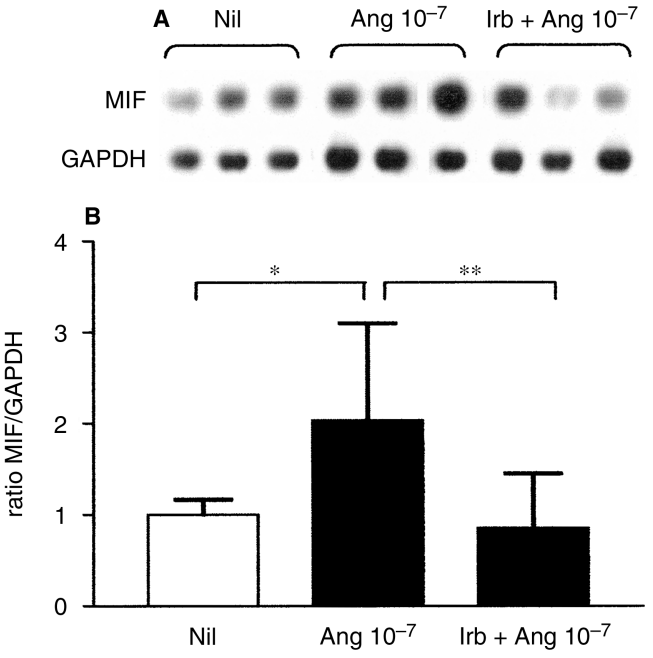

Fig. 7. Angiotensin II (Ang II) stimulates macrophage migration inhibitory factor (MIF) mRNA expression in MCT cells, which is blocked by irbesartan (Irb). (*A*) Northern blot in which MCT cells were either left unstimulated (Nil), stimulated with 10^{-7} mol/L Ang II or incubated with 10^{-4} mol/L Irb for 1 hour prior to stimulation with 10^{-7} mol/L Ang II (Irb + Ang 10^{-7}) for 6 hours. Blots were probed for MIF and then reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (*B*) Results of densitometric analysis of the combined results of 3 experiments are shown. Data are mean ± SD. $*P < 0.05$; $**P < 0.01$, by ANOVA with Bonferroni's post-test comparison.

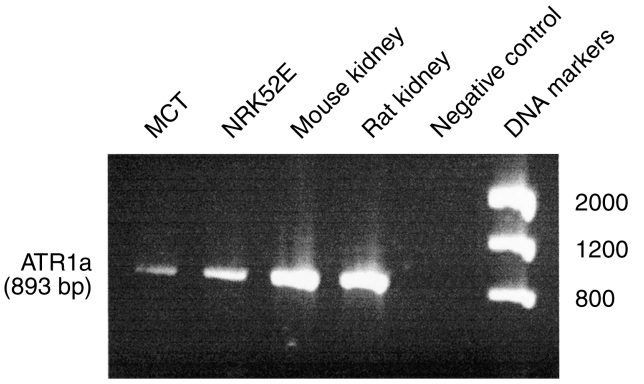

Fig. 8. NRK52E and MCT express the angiotensin II (Ang II) receptor 1a subtype (ATR1a). RNA was extracted from NRK52E and MCT cells, reverse transcribed and then ATR1a detected by polymerase chain reaction (PCR). A single band of the expected 893 bp for ATR1a was detected in both cell lines. Positive controls were normal mouse and rat kidney. Negative control omitted the reverse transcripton (RT) product. DNA markers of 800, 1200, and 2000 bp are shown.

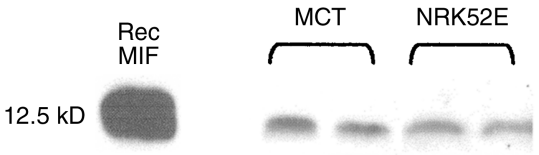

Fig. 9. Constitutive macrophage migration inhibitory factor (MIF) protein expression by tubular epithelial cell lines. Western blotting of MCT and NRK52E cell lysates demonstrates constitutive expression of a protein band at 12.5 kD that corresponds in size to recombinant human MIF (Rec MIF).

phage ($r^2 = 0.35$, $P < 0.01$) and T-cell infiltration ($r^2 = 0.22$, $P < 0.05$).

Ang II induces up-regulation of MIF mRNA in vitro

The ability of irbesartan to prevent up-regulation of tubular MIF expression in STNx could be due to a direct effect of Ang II on MIF gene expression, or an indirect effect of suppression of renal injury. Therefore, we examined whether Ang II has a direct effect on MIF expression by studying tubular epithelial cells in vitro. Cultured NRK52E tubular epithelial cells constitutively express MIF mRNA. Ang II (10^{-5} or 10^{-6} mol/L) increased MIF mRNA levels twofold within 6 hours and could be prevented by prior addition of 10^{-4} mol/L irbesartan. 10^{-7} mol/L Ang II did not cause an increase in expression of MIF mRNA (Figs. 5 and 6). These findings were confirmed in a second tubular cell line. MCT cells were also shown to constitutively express MIF mRNA and the addition of 10^{-7} mol/L Ang II doubled MIF mRNA levels. This effect was also completely prevented by preincubation with 10^{-4} mol/L irbesartan (Fig. 7). PCR analysis showed that both NRK52E and MCT cell lines express the Ang II receptor 1a subtype (Fig. 8).

Ang II induces secretion of MIF in vitro

Analysis by Western blotting and flow cytometry revealed that unstimulated NRK52E and MCT cells contain MIF protein (Figs. 9 and 10). Stimulation of NRK-52E cells with Ang II 10^{-5} mol/L caused rapid secretion (within 20 minutes) of approximately 50% of MIF protein stores from cells as shown by a loss of intracellular MIF protein staining detected by flow cytometry. Levels of intracellular MIF returned to baseline over the next 2 hours (Fig. 10). Addition of irbesartan prevented Ang II–induced MIF secretion by NRK52E cells (Fig. 10). Confirmation of MIF secretion from NRK52E cells was performed by detection of increased amounts of MIF in the cell culture media by immunoprecipitation and Western blotting. This showed that MIF is constitutively secreted by NRK52E cells and that stimulation with Ang II for 20 minutes caused increased accumulation of MIF protein in the media (Fig. 11).

DISCUSSION

The RAS has a pathogenic role in immune- and non-immune-mediated renal diseases in humans and experimental models. Some of the beneficial effects of Ang II

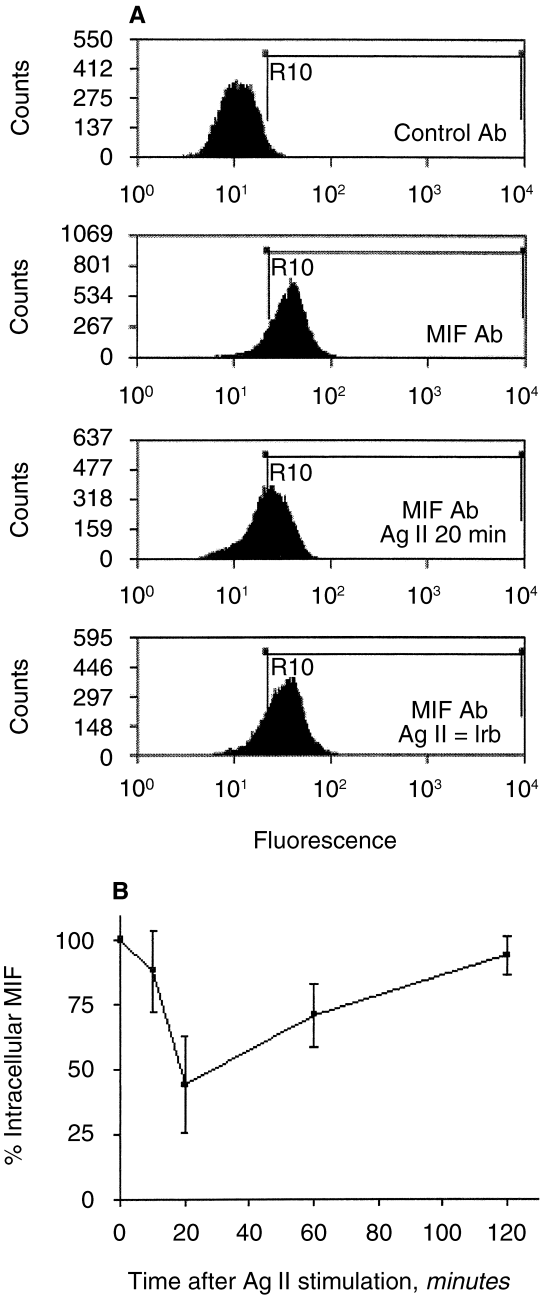

Fig. 10. Flow cytometry analysis of intracellular macrophage migration inhibitory factor (MIF) protein. NRK52E cells were stimulated with 10^{-5} mol/L angiotensin II (Ang II) for various periods, fixed, permeabilized, and labeled with Alexa 488–conjugated OX-8 (Control Ab) or Alex-conjugated IIID9 (MIF Ab). (*A*) Histograms from one experiment show baseline MIF expression (MIF Ab) compared to the negative control antibody (Control Ab). Twenty minutes after Ang II stimulation there is a marked reduction in MIF protein within the cells, which is largely prevented by the presence of 10^{-4} mol/L irbesartan. (*B*) The combined results of three separate experiments. Data are mean ± SD. $^*P < 0.05$ vs. unstimulated cells by ANOVA with Bonferroni's post-test comparison.

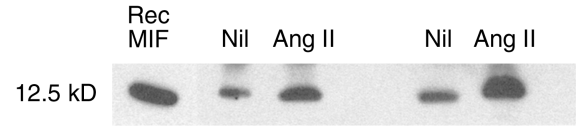

Fig. 11. Secretion of MIF protein by NRK52E cells is increased by angiotensin II. Macrophage migration inhibitory factor (MIF) was immunoprecipitated from the supernatant of unstimulated (Nil) NRK52E cells or NRK52E cells stimulated with 10^{-5} mol/L angiotensin II (Ang II) for 20 minutes, and then detected by Western blotting using a MIF antibody. This revealed secretion of a 12.5 kD protein by NRK52E cells that was the same size as recombinant human MIF protein (Rec MIF).

blockade by ACEI or AT_1RA are independent of blood pressure control. Ang II regulates a variety of inflammatory cell responses, including mononuclear cell chemotaxis [10, 39] and proliferation [40], as well as cytokine production by inflammatory cells [6]. It is unknown if these effects on leukocytes are a direct effect of Ang II or whether they occur through induction of other proinflammatory molecules. This study provides evidence that Ang II–mediated macrophage and T-cell accumulation and activation may occur indirectly through MIF.

STNx in rats is a model of progressive renal injury characterized by infiltration of macrophages into the glomerulus and interstitium that leads to secondary focal and segmental glomerulosclerosis and tubulointerstitial damage [41, 42]. In the STNx model, there is local activation of the RAS within the kidney with evidence of de novo expression of renin and Ang II in tubules. There are several studies showing reversal of leukocyte infiltrate and renal damage in this disease model by the use of ACEI or AT_1RA [43–45]. We hypothesized that local activation of the tubular RAS plays a central role in inducing increased expression of MIF that results in leukocyte-mediated renal injury.

In this study we showed that MIF mRNA was upregulated in tubular and glomerular epithelial cells following STNx. Blockade of the RAS with irbesartan, an AT_1RA, reduced MIF mRNA expression in tubular and glomerular epithelial cells. Reduction in MIF expression correlated with reduced macrophage and T-cell infiltrate, suggesting that Ang II may stimulate renal injury indirectly through MIF.

The up-regulation of MIF expression in this model may be due to a direct effect of Ang II or an indirect effect through the action of other factors such as hypertension. Therefore, to examine the relationship between Ang II and MIF, we performed in vitro experiments in two tubular epithelial cell lines. We found that Ang II doubled MIF mRNA expression and this could be prevented by irbesartan. This provided evidence that the proinflammatory activities of Ang II may be mediated through the induction of MIF synthesis in renal tubular epithelial cells via activation of the Ang II type I receptor.

Local, rather than systemic production, of both Ang II and MIF by the kidney is important in renal disease. Dissociation between intrarenal and plasma Ang II levels has been shown in several animal models such as STNx [46], two-kidney, one clip Goldblatt hypertension [47], Ang II–induced hypertension [48], and hyperten-

sive ren-2 transgenic rats [49]. Similarly, serum MIF levels are not elevated in humans with glomerulonephritis or renal transplant rejection while immunohistochemistry reveals up-regulation of MIF in the kidney [50, 51]. This suggests that understanding the mechanisms of local production of Ang II and MIF and release of these molecules by renal cells is important in increasing our knowledge on the pathogenesis of renal disease. In this study, we found that Ang II causes secretion of MIF by tubular epithelial cells in vitro. Therefore, local production of Ang II by mesangial cells or macrophages following a renal insult may lead to MIF secretion from tubular epithelial cells and thereby amplify further macrophage and T cell activation and consequently promote renal damage.

Up-regulation of MIF may not be the sole mechanism by which Ang II exerts its proinflammatory effects in this model. Renal cortical levels of mRNA for endothelial cell adhesion molecules [intercellular adhesion protein (ICAM)-1, vascular cell adhesion molecule (VCAM)-1] as well as cytokines [interleukin (IL)-1β, osteopontin] and chemokines [monocyte chemoattractant protein (MCP)-1] are also up-regulated in this model [52–54]. The increased expression of these molecules can be prevented by the administration of ACEI or AT_1RA suggesting that STNx causes an Ang II–mediated up-regulation of several molecules that recruit or activate macrophages. In addition, Ang II has been shown in vitro to directly up-regulate MCP-1 in mesangial cells [8] and ICAM-1 in proximal tubules [55].

As well as providing insight into a possible mechanism by which Ang II may regulate its proinflammatory effects in renal disease, this study has also provided further information on factors that regulate MIF production and secretion. MIF is up-regulated in many types of renal disease [20, 56, 57] and has been shown to have a pathogenic role in rat crescentic glomerulonephritis [29, 30]. We have previously shown that tumor necrosis factor (TNF)-α increases MIF mRNA expression in tubular epithelial cells in vivo [58], while, in vitro, platelet-derived growth factor (PDGF)-AB and interferon (IFN)-γ up regulate MIF mRNA in mesangial cells [38]. This study has provided evidence that Ang II up-regulates tubular epithelial cell expression of MIF mRNA in vivo and in vitro. This finding is supported by the observation that MIF mRNA expression was up-regulated by Ang II in neurons of the hypothalamus and brain stem in vitro [59].

CONCLUSION

The RAS plays a central role in the pathogenesis of chronic renal disease. Much experimental data now suggest that Ang II is involved in several steps of the inflammatory response as well as being a vasoactive hormone. Up regulation of MIF mRNA and MIF protein secretion may be an important mechanism by which Ang II recruits and activates inflammatory cells, which results in renal damage.

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